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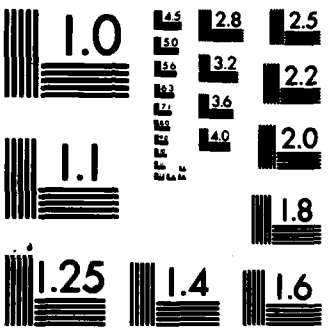

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# Isolation and Characterization of Erythrocyte and Parasite Membranes from Rhesus Red Cells infected with P. Knowlesi

Final Report

Donald F. Wallach, M.D.

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Erythrocytic forms of Plasmodia synthesize glycoproteins that become exported from the parasite and its vacuole to be inserted into the host cell membrane. The best characterized protein of this category is an $M_r$ 74 000, pI 4.8 glycoprotein, synthesized by P. knowlesi, PkGP74, has immunogenic glycopeptide exposed at the external host cell membrane surface of erythrocytes infected with P. knowlesi schizonts, Presence of antibodies against PkGP74, correlate with immune protection of monkeys against P. knowlesi. PkGP74 shares peptide and immunogenic moieties with a similar protein located in the membranes of erythrocytes infected with P. falciparum.

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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).


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## Biochemical characterization of purified P. knowlesi SI-RBC membrane antigens

We have adapted technology originally applied to the isolation and protein characterization of uncontaminated plasma membranes from normal and neoplastic lymphocytes, keeping balance sheets of markers for surface and intracellular components. Lactoperoxidase-catalyzed surface radioiodination of intact normal erythrocytes and P. knowlesi schizont-infected red blood cells, SI-RBCs, was used to mark protein segments at the external surface. As P. knowlesi infections are synchronized, monitoring of blood smears permits marking and processing before the host cell membrane breaks down during merozoite release. This allows one to restrict the label to cell surface components exclusively. We carry out cell disruptions and fractionations at high speed, high dilution and in the cold to minimize aggregation and proteolysis. Monitoring the appearance of low $M_r$ components with time by DS-PAGE, shows that proteolysis artefacts are not significant under our conditions and are not changed by protease inhibitors.

SI-RBCs are disrupted by nitrogen decompression and fractionated centrifugally into schizonts and small, sealed, normally oriented SI-RBC surface membrane vesicles. Analysis, using DS-PAGE, of P. knowlesi SI-RBC membranes after whole cell surface radioiodination showed conventional erythrocyte membrane protein patterns, except for the addition of readily distinguished 'neocomponents' with a wide range of $M_r$s. Labeling of cytoplasm proteins was negligible, indicating non-penetration of the labeling reagent. Extended incubation of membranes (or homogenate) at 20° produced no DS PAGE evidence for proteolysis.

Comparison, using two-dimensional isoelectric focusing/DS PAGE, (IEF/DS PAGE, of normal and SI-RBC membranes showed appearance in the latter of several glycoproteins, most prominently proteins with $M_r$s of 74 000 and 102 000, GP74 and GP102. These proteins occur at only low levels in isolated schizonts. IEF/DS PAGE analysis after surface radioiodination showed that both GP74 and GP102 are exposed at the external surface of the SI-RBC. Metabolic labeling of P. knowlesi SI-RBC with $^{14}C$-amino acids, $^{35}S$ methionine, and $^{14}C$-carbohydrate precursors, under conditions avoiding label reutilization marked both GP102 and GP74, indicating the parasite origin of both peptide and saccharide moieties. No labeling was obtained with uninfected erythrocytes.

## Immunochemistry

Antibodies were obtained from rhesus monkeys vaccinated with P. knowlesi SI-RBCs, animals infected with P. knowlesi SI-RBC, cured with chloroquine and reinfected twice, and Gambian individuals immune to P. falciparum. Analyses by crossed immune electrophoresis and immune precipitation, using necessary controls, showed that GP102 and GP74 were accessible at SI-RBC surfaces to react with antibodies. Further evaluation showed that protective immunity correlates only with high titres of GP74. The sera precipitate proteins of equivalent $M_r$s and pIs from membranes of P. cynomolgi-infected rhesus erythrocytes. GP74 is deposited also by sera of humans immune against P. falciparum malaria. Related interspecies reactivity occurs between extracts of P. falciparum infected human erythrocytes and purified P. knowlesi merozoites.

## Subcellular fractionation of parasitized erythrocytes

As P. falciparum induces only partially synchronized infections in squirrel monkeys, our fractionation procedure for P. knowlesi was modified. To reduce contamination of infected erythrocytes by leukocytes, splenectomized animals were irradiated with 300 rad three times in ten days prior to bleeding. Blood was drawn at a parasitemia between 40 and 70% (routinely attained in splenectomized squirrel monkeys) and residual leukocytes reduced to less than 0.05% by a 1.08 density Ficoll/Hypaque gradient. SI-RBCs were collected at more than 80% yield atop a 1.087 Ficoll/Hypaque density barrier. Surface-labeling was by lactoperoxidase-catalyzed radioiodination. For nucleic acid labeling freshly isolated, parasitized erythrocytes in RPMI-1640 (10% dialyzed, heat-inactivated host serum; hematocrit of 6-10%) were incubated with $^{3}$H-orotic acid (0.05 mCi/ml). After labeling, SI-RBCs were disrupted and fractionated as before. We recovered about 50% of total $^{125}$I-activity associated with the SI RBC surface in the host cell plasma membrane fraction, with less than 5% membrane contamination of the purified parasites released from infected erythrocytes. Conversely, more than 70% of $^{3}$H-orotic acid labeling co-purified with parasites, with not more than 7% of nucleic acid material associated with the isolated membrane. The results are comparable to those for P. knowlesi SI-RBCs.

Ring and early trophozoite-stages were isolated at a high yield and purity as follows: Cells pelleting through density 1.087 were disrupted by nitrogen decompression at 600 psi for 15-20 min. This releases the parasites and converts the erythrocyte membranes into small vesicles. After a short exposure to hypotonic buffer, the higher-density early-stage vesicles and the low-density vesicles were separated using a 1.06 density Ficoll/Hypaque density barrier, through which the parasites sediment quantitatively. According to $^{125}$I-labeling, about 75% of the covalently bound isotope was recovered in the membrane fraction, 7% was associated with the parasite pellet. The residual activity was found in the supernatant after pelleting of membrane vesicles at $2.10^{7}$ g·min. More than 90% of the label from tritiated orotate fractionated with the parasites and less than 5% was associated with the membrane fraction.

## Metabolic labeling of intraerythrocytic plasmodial parasites.

We use $^{35}$S-methionine for metabolic labeling of the intraerythrocytic stages of P. knowlesi and P. falciparum. After removal of leukocytes, packed rhesus or squirrel monkey erythrocytes, infected with P. knowlesi or P. falciparum, respectively, were injected into methionine-free RPMI 1640 containing 0.2 mCi/ml $^{35}$S-methionine (400 Ci/mmol) and supplemented with dialyzed heat-inactivated serum of the respective host species (hematocrit - 10%). The medium was pre-equilibrated at 5.3% $O_2$, 5% $CO_2$ and 89.7% $N_2$. Metabolic labeling was for 3 h. The specific $^{35}$S-methionine activity of the purified parasites was 3 - 8 x $10^{7}$ cpm/mg protein and that of infected membrane 2-6 x $10^{6}$ cpm/mg protein. Parasite nucleic acids were labeled under the same culture conditions with $^{3}$H-orotic acid (0.05 mCi/ml medium) for 3 h. Uninfected erythrocytes from the same cell preparation (assuming a similar concentration of reticulocytes) were incubated with either precursor under identical conditions incorporated less than 0.02% of the isotope incorporated by parasitized erythrocytes.

We have pulse-labeled P. knowlesi SI-RBCs using high specific activity $^{14}C$-glucosamine. Two-hour labeling at an isotope concentration of 0.2 mCi/ml followed by a chase with 2 mM unlabeled glucosamine led to incorporation of label into parasite-synthesized host cell membrane proteins with $M_r$s between 230 000 and 40 000.

## Cell-free translation

We have initiated studies to isolate and characterize the mRNA of P. knowlesi, translating the parasite messenger by use of reticulocyte lysates. For the isolation of mRNA we used purified late stage P. knowlesi SI-RBCs. Contamination of the infected red blood cells by leukocytes was less than 0.01%. Cell pellets were frozen in NaCl/TrisHCl (130 mM/10mM), pH 7.5 in liquid nitrogen and stored at -70°. Pellets were resuspended in ice in 10 ml of prechilled lysis buffer - NaCl/TrisHCl/$MgCl_2$/Nonidet P40 (150 mM/20mM/ 1 mM/0.65%, v/v) [pH 8.0], containing adenosine-vanadyl complex (16 mM). After addition of DS and NaCl to final concentrations of 0.5% (v/v) and 300 mM, respectively, the mixture was extracted five times with equal volumes of phenol containing 0.2% (w/v) 8-hydroxyquinoline, followed by one extraction with chloroform/isoamyl alcohol, two ether extractions, and precipitation with three volumes of ethanol. The precipitate was washed twice in ethanol/$H_2O$ (70/30; v/v), dried in vacuo, and redissolved in autoclaved deionized water. The yield averaged 0.7 mg. nucleic acid per $10^{10}$SI-RBCs.

Aliquots of RNA were chromatographed on oligo-(dT) cellulose in NaCl/-Tris-HCl/EDTA/DS, (400 mM/10 mM/10 mM/0.1%, w/v), [pH 7.5]. Oligo-(dT)-bound RNA was eluted in sterile deionized water. P. knowlesi has poly-(A) tails, as evidenced by its affinity for oligo-(dT)-cellulose. Between 2 and 6% of the applied RNA bound to oligo-(dT)-cellulose and therefore represents mRNA. Oligo-dT-cellulose-bound plasmodial RNA directed the in vitro translation of all the proteins coded for by the total RNA. However, mRNAs coding for proteins with $M_r$s greater than 100 000 bound poorly in 400mM NaCl. This presumably reflects the large size of the message compared with the small poly(A) tail.

In vitro culture of P. knowlesi schizonts and cell-free translation of P. knowlesi mRNA, produced parasite proteins with a wide range of $M_r$s. Both systems yielded prominent components with $M_r$s of 230 000, 170 000, 140 000, 102 000, 74 000, 52 000, 46 000, 40 000, and 30 000.

Our results with metabolically labeled parasite protein confirm our findings that the membranes of P. knowlesi SI-RBCs contain parasite-synthesized proteins with $M_r$s of 140 000 and 102 000 that appear slightly smaller than the equivalent proteins in the parasite. GP74 isolated from the membranes gives a broader band on DS PAGE than the corresponding $M_r$ 74 000 protein from parasites.

Cell-free translation produced significant quantities of proteins with $M_r$s between 230 000 and 20 000 within 20 min. Synthesis was stable for 200 min. Major components with $M_r$s near 140 000, 102 000 and 74 000 appear to correspond to proteins synthesized by the intraerythrocytic parasite.

The antigenic similarity of P. knowlesi components produced by metabolic labeling and in vitro translation was investigated by an antigen competition experiment: $^{35}S$-methionine-labeled translated proteins were reacted with rhesus immune Ig in the presence of increasing concentrations of P. knowlesi antigen from SI-RBCs. Antigens from both the parasite and host cell membrane

inhibited the specific precipitation of proteins translated in vitro. This indicates that the proteins translated in vitro share antigenic determinants with the parasite-synthesized proteins. Competition was most impressive for the $M_r$ 140 000 and $M_r$ 74 000 components, when host cell membranes were used as antigen.

Immunological reactivity of proteins by cell-free translation of P. knowlesi mRNA and from metabolically labelled P. knowlesi SI-RBC.

The immunological reactivity of P. knowlesi antigens produced by cell-free translation was tested for by immune precipitation with rhesus monkey immune sera. The precipitated components were compared to those deposited from P. knowlesi schizonts metabolically labeled in vitro.

Rhesus immune sera precipitated components with $M_r$s near 230 000, 140 000, 125 000, 74 000 and 42 000. The $M_r$ 230 000 and 125 000 components had not been identified with $^{125}I$-labeling and the $M_r$ 74 000 appeared to be more prominent when $^{125}I$-labeled antigen was employed for immune precipitation. However, we found that $^{125}I$-labeling can cause partial degradation of the $M_r$ 230 000 and $M_r$ components leading to relative increase in the 74 000 protein.

$M_r$ 140 000, 102 000, 74 000 components were consistently precipitated from the translation mixture. The data point to a homology between the $M_r$ 74 000 protein produced by the parasite and the protein synthesized by cell-free translation. Significantly, the translated $M_r$ 74 000 protein reacts with antibodies in sera of squirrel monkeys immune against P. falciparum infections.

In vitro activity of antiplasmodial antibodies

We have extended our observation on the specific immune reactivity of P. knowlesi and cross-reacting P. falciparum antigens with sera of rhesus monkeys and Gambian individuals immune against P. knowlesi and P. falciparum malaria, respectively. The cytotoxic effects of Ig from immune and non-immune rhesus monkeys were tested across plasmodial species in cultures of P. falciparum in human erythrocytes. The P. falciparum cultures were maintained in microtiter plates at a hematocrit of 6% in RPMI 1640/10% heat inactivated human serum. Parasite multiplication under standard conditions was compared to that in presence of 5 and 10 ul of Ig (equivalent to 5 and 10 ul of serum) from rhesus monkeys unexposed to or protected against P. knowlesi infections. During 72 hr. a 20-fold multiplication was commonly observed for control cultures and cultures containing normal rhesus Ig. In contrast, addition of immune serum suppressed total parasitemia after 72 h by more than 50%. This inhibition of P. falciparum multiplication was highly significant ($p$ less than 0.001). Differential counts of the parasite stages in asynchronous cultures or cultures synchronized by the sorbitol technique indicated a significant delay in parasite maturation. There was a more than 3-fold higher absolute concentration of SI-RBCs/well in comparison to the two sets of control cultures. The data suggests that, in addition to inhibiting reinvasion, Ig from immune monkey serum compromises intraerythrocytic maturation of the parasite.

The above studies have been extended and made more specific by the use of monoclonal antibodies that react solely with the GP74 or the $M_r$ 230 000

protein from the membranes of P. knowlesi SI-RBCs. Monoclonal antibodies were precipitated with $(NH_4)_2SO_4$ and adjusted to the Ig concentration of 0.5 mg/ml used for the in vitro testing of growth-inhibitory activity. P. knowlesi cultures in rhesus erythrocytes were initiated from freshly drawn monkey blood (at a parasitemia near 2%; schizonts) and maintained for 36 hrs. at a hematocrit of 6% in RPMI 1640/10% rhesus serum, using 96 well microtitre plates and candle jar conditions. P. falciparum in human erythrocytes was maintained similarly for 72 h in RPMI 1640/10% human serum. Monoclonal antibody against GP74 significantly retarded the intraerythrocytic maturation of the P. knowlesi and caused a 60-70% decrease in parasitemia at 36 hrs. The antibody against the $M_r$ 230 000 protein has no effect on parasite maturation but significantly suppressed parasitemia after completion of the first cycle (between 24 and 36 hrs.). This suggests an action on the late schizont or merozoite stages. Antibody against GP74 exhibited a growth-inhibitory effect on P. falciparum in human erythrocytes similar to but quantitatively less than that found with antibodies from the serum of monkeys immune against P. knowlesi. The antibody against the $M_r$ 230 000 component showed no activity across plasmodium species lines.

The results indicate that some plasmodial proteins on the surfaces of SI-RBCs are accessible to Ig binding and may represent the target for antibody-mediated, spleen-independent parasite growth inhibition. The data confirm previous results indicating that P. knowlesi GP74 shares antigenic determinant with P. falciparum.

Membrane disposition of GP74

We are developing ways to determine the transmembrane disposition of parasite-synthesized proteins in SI-RBC membranes. As this will involve radiolabeling from the outside and inside surfaces of the host cell membrane, we have tested the feasibility of preparing normally oriented and inverted membrane vesicles. Primary membrane vesicles obtained by disrupting SI-RBCs were diluted 30-fold in 10 mM HEPES, pH 8.0, washed, resuspended in 0.5 mM HEPES, pH 8.0 and washed again. After passage through a 27 gauge needle the inverted vesicles were resealed by increasing ionic strength and returning to pH 7.4. According to assays of acetylcholinesterase and NADH: cytochrome c reductase in the presence or absence of 0.2 % Triton X-100, approximately 50-60% of the membrane vesicles were inside-out. Inside-out vesicles can be quantitatively separated from normal vesicles by flotation on 1.09 density dextran, to give a population of about 85-90% inside-out vesicles.

Transmembrane disposition will be ultimately determined by tryptic peptide mapping. In preliminary work intact SI-RBCs were surface-labeled using lactoperoxidase-catalyzed radioiodination. GP74 was isolated by DS-PAGE after immune precipitation and subjected to digestion by TPCK-trypsin using conditions described above. The $^{125}I$-peptides were fractionated by HPLC and bidimensional thin layer electrophoresis/chromatography. In both systems only 4 of 14 peptides were labeled, representing the polypeptide moieties exposed at the cell surface. This information confirms previous results indicating that GP74 is exposed at the surface of the host cell membrane, and identifies the surface exposed peptide moieties.

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